EXHIBIT B

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[DOCUMENT NAME] CLAIMS

[CLAIM 1]
A recombinant virus vector that originates in HHV-6 and includes an exogenous nucleotide sequence in a portion corresponding to at least one region selected from the group consisting of U2, U3, U4, U5, U6, U7, and U8 regions of HHV-6.

[CLAIM 2]
A recombinant virus vector as set forth in claim 1, wherein, said portion exists between nucleotide numbers 10216 and 16547 of a HHV-6 DNA sequence as represented by SEQ ID NO: 1.

[CLAIM 3]
A recombinant virus vector as set forth in claim 1, which comprises H6R28 virus.

[CLAIM 4]
A recombinant virus vector that originates in HHV-7 and includes an exogenous nucleotide sequence in a portion corresponding to at least one region selected from the group consisting of U2, U3, U4, U7, and U8 regions of HHV-7.

[CLAIM 5]
A recombinant virus vector as set forth in claim 4, wherein, said portion exists between nucleotide numbers 10558 and 18483 of a HHV-7 DNA sequence as represented by SEQ ID NO: 2.

[CLAIM 6]
A recombinant virus vector as set forth in claim 4, which comprises H7R28 virus.

[CLAIM 7]
A recombinant virus vector as set forth in claim 1 or 4, wherein, the exogenous nucleotide sequence is a DNA sequence and/or RNA sequence.

[CLAIM 8]
A recombinant virus vector as set forth in claim 7, wherein, the exogenous nucleotide sequence encodes at least one kind of substance selected from the group consisting of a bacterial artificial chromosome (BAC), cytokine gene, ribozyme, interference RNA, immunological co-stimulator molecule, signal transduction molecule, enzyme, and chemical attractant.

[CLAIM 9]
A recombinant virus vector as set forth in claim 7, wherein, the exogenous nucleotide sequence is used for gene therapy of mammals.

[CLAIM 10]
A recombinant virus vector as set forth in claim 7, wherein, the exogenous nucleotide sequence includes a nucleotide sequence that encodes a marker gene.

[CLAIM 11]
A producing method of a recombinant virus vector that originates in HHV-6,
said method comprising the step of inserting an

exogenous nucleotide sequence in a portion corresponding to at least one region selected from the group consisting of U2, U3, U4, U5, U6, U7, and U8 regions of HHV-6.

[CLAIM 12]
A producing method of a recombinant virus vector as set forth in claim 11, wherein, in the step of inserting an exogenous nucleotide sequence, the exogenous nucleotide sequence is inserted between nucleotide numbers 10216 and 16547 of a HHV-6 DNA sequence as represented by SEQ ID NO: 1.

[CLAIM 13]
A producing method of a recombinant virus vector as set forth in claim 11, wherein, in the step of inserting an exogenous nucleotide sequence, homologous recombination is carried out between a HHV-6 DNA sequence and a DNA sequence that is amplified with a set of primers of sequences represented by SEQ ID NO: 3-4 and SEQ ID NO: 5-6.

[CLAIM 14]
A producing method of a recombinant virus vector that originates in HHV-7,
said method comprising the step of inserting an exogenous nucleotide sequence in a portion corresponding to at least one region selected from the group consisting of U2, U3, U4, U7, and U8 regions of HHV-7.

[CLAIM 15]
A producing method of a recombinant virus vector as set forth in claim 14, wherein, in the step of inserting an

exogenous nucleotide sequence, the exogenous nucleotide sequence is inserted between nucleotide numbers 10558 and 18483 of a HHV-7 DNA sequence as represented by SEQ ID NO: 2.

[CLAIM 16]
A producing method of a recombinant vector as set forth in claim 11 or 14, wherein, in the step of inserting an exogenous nucleotide sequence, the exogenous nucleotide sequence is inserted inside a normal cell and/or an umbilical cord blood cell.

[CLAIM 17]
A transforming method of a host cell, wherein the method transforms a host cell of a mammal with a recombinant virus vector of claim 1 or 4,
said method comprising the step of transforming, with the recombinant virus vector, a host cell of at least one kind of mammal selected from the group consisting of a human, a non-human primate, and a host that is open to HHV-6 or HHV-7 infection.

[CLAIM 18]
A transforming method of a host cell as set forth in claim 17, wherein, in said step of transforming a host cell, the method transforms, with the recombinant virus vector, at least one kind of host cell selected from the group consisting of a T cell, macrophage, glial cell, peripheral-blood mononuclear cell, blood stem cell, liver cell, fibroblasts, and natural killer cell.

[CLAIM 19]

A transformed host cell, which is obtained by the transforming method of any one of claims 16 through 18.

[CLAIM 20]
A transformed host cell as set forth in claim 19, which is used for gene therapy of mammals.

[CLAIM 21]
A transformed host cell as set forth in claim 20, wherein the gene therapy is (i) for preventing human immunodeficiency virus (HIV) infection of a compromised cell caused by HIV, and/or (ii) for immunotherapy of cancer.

[CLAIM 22]
A transformed host cell as set forth in claim 20, wherein the host cell is derived from a mammal of the kind subjected to the gene therapy.

[CLAIM 23]
A gene therapy method for non-human mammals, comprising the step of administering a transformed cell of any one of claims 19 through 22 to the mammal.

[CLAIM 24]
A gene therapy method for non-human mammals, comprising the step of transforming, with a recombinant virus vector of claim 1 or 4, a host cell of a mammal in vivo at a multiplicity of infection (MOI) of 0.01 to 20.

[CLAIM 25]
A gene therapy method as set forth in claim 23 or 24,

comprising the step of expressing a gene encoded by the exogenous nucleotide sequence included in the recombinant virus vector.

[DOCUMENT NAME] SPECIFICATION

[TITLE OF THE INVENTION]

RECOMBINANT VIRUS VECTOR ORIGINATING IN HHV-6 OR HHV-7, METHOD OF PRODUCING THE SAME, METHOD OF TRANSFORMING HOST CELL USING THE SAME, HOST CELL TRANSFORMED THEREBY AND GENE THERAPY METHOD USING THE SAME

[TECHNICAL FIELD]

[0001]

The present invention relates to a recombinant virus and a recombinant virus vector, and more specifically to a recombinant virus, and a recombinant virus vector, originating in HHV-6 and HHV-7, which are members of the herpesvirus.

[0002]

The present invention also relates to a producing method of such a recombinant virus and recombinant virus vector. The invention also relates to a method of transforming a host cell using such a recombinant virus and recombinant virus vector. Further, the invention relates to a host cell transformed by such a recombinant virus and recombinant virus vector. The invention also relates to a gene therapy method using such a recombinant virus and recombinant virus vector.

[BACKGROUND ART]

[0003]

Accumulation of knowledge and various technological advances in molecular biology and molecular genetics have greatly contributed to the recent progress in life science, providing rich information on various living

phenomena.

[0004]

Currently, there have been ongoing active research and development in various fields of life science, with particular interest in the analysis of gene functions. This has led to the development of techniques and vectors for introducing isolated genes into cells and individual living organisms.

[0005]

For medical applications, there have been developed various types of vectors used to introduce genes into mammalian cells. Among these vectors, vectors using viruses (virus vectors) have drawn many interests.

[0006]

Virus vectors have advantages over other known genes in introducing a foreign gene into a cell for protein expression. The central idea underlying the gene transfer using the virus vector is to introduce a foreign gene into an infected cell and transform the cell with the foreign gene under control of promoter sequences, taking advantage of the infectious capacity of the virus (productive infection, latent infection, abortive infection).

[0007]

Conventional transfection techniques include non-viral methods. Examples of non-viral methods include: simple addition of a target gene construct as free DNA; incubation with a complex of target DNA and a specific protein that is designed to uptake the DNA into a target cell; and incubation with target DNA that is contained in infected genes that are encapsulated by liposome and other lipids. However, these non-viral transfection techniques suffer from poor efficiency, and the expression

efficiency of introduced genes is generally poor.

[0008]

One conventional transfection technique uses recombinant viruses, and recombinant virus vectors, that are manipulated to include essential target genes, can infect target cells, and therefore enables the target genes to be expressed in the cells. Various types of viruses, such as retrovirus, adenovirus, and adeno-associated virus are used for this purpose. However, these viruses have the following drawbacks.

[0009]

For example, the retrovirus is carcinogenic, and its carcinogenicity in a gene therapy has been reported. Another drawback of the retrovirus is that it can incorporate only small genes and is selective as to the types of cells that can be used to express the genes.

[0010]

As to the adenovirus, it can trigger a strong allergic reaction when used in a gene therapy or the like. Further, the adenovirus suffers from poor efficiency when used to introduce genes into blood cells. It is therefore difficult to use the adenovirus as a vector.

[0011]

The adeno-associated virus allows for introduction of only small genes, and its gene expression efficiency is poor. Another drawback of the adeno-associated virus is that it is difficult to produce a vector. Further, there is a potential risk of causing cancer when incorporated in the host gene.

[0012]

To this date, eight broad kinds of viruses have been identified that belong to the herpesvirus family, taking

into account only those that are infectious. The herpesvirus is a large DNA virus, and is broadly classified into three sub families α, β, and γ according to the phylogenetic tree, with distinct biological characteristics in each sub family. For example, α-herpesvirus is a neurotropic virus that exhibits latency and reactivation in nerve cells, whereas γ-herpesvirus is oncogenic.

[0013]

Human β-herpesvirus includes human cytomegalovirus (HCMV: human herpesvirus 5, HHV-5), human herpesvirus 6 (HHV-6), and human herpesvirus (HHV-7).

[0014]

Of these viruses, HHV-6 and HHV-7 in particular have drawn many interests as the candidates for virus vectors used for gene therapy (see Non-Patent Citation 2, for example), since the disease causes by these viruses shows mild symptoms (see Non-Patent Citation 1, for example).

[0015]

Using the herpesvirus, and HHV-6 and HHV-7 in particular as a recombinant virus and a recombinant virus vector has certain advantages, which include low pathogenicity, ease of gene introduction into blood cells such as the T cell and macrophage, and introduction of relatively large genes.

[0016]

Using HHV-6 as a recombinant virus or a recombinant virus vector is advantageous in the following respects. First, it allows for gene introduction into a macrophage, which is difficult with other vectors. Further, since the gene can be introduced into the macrophage in latency, the allergic reaction seen with the adenovirus does not occur.

[0017]

However, it is difficult to produce a recombinant virus, and a recombinant virus vector, that originates in HHV-6 or HHV-7, and, today, no method is available that can produce such viruses and vectors. One of the factors that makes recombination of HHV-6 and HHV-7 difficult, beside technical factors, can be attributed to the characteristics of HHV-6 and HHV-7 genes.

[0018]

The size of gene in HHV-6 and HHV-7 is smaller than that in HCMV, and HHV-6 and HHV-7 contain essentially no genes that are dispensable for the viral replication as observed in HCMV (see Non-Patent Citations 3 and 4, for example).

[0019]

As a rule, use of a homologous recombination method to produce a recombinant virus or a recombinant virus vector of the herpesvirus requires destruction of one or more sites. However, the recombination sites that have been conventionally used for the preparation of HCMV recombinant viruses are not necessarily included in HHV-6 and HHV-7. Accordingly, development of a new method is needed for the preparation of a recombinant virus and a recombinant virus vector of HHV-6 and HHV-7.

[0020]

As a virus vector originating in the herpesvirus, there has been proposed a foreign gene that is inserted in the genome of a herpes simplex virus under control of a promoter regulating region of the genome, and therefore serving as a vector for expressing foreign genes (see Patent Citation 1, for example). There are also disclosed a

DNA construct, a plasmid vector including a construct useful for the expression of foreign genes, a recombinant virus produced by such a vector, and methods concerning these. However, these publications merely describe a herpes simplex virus type 1 (HDV-1) vector and a producing method thereof, and do not disclose anything about virus vectors originating in HHV-6 or HHV-7.

[0021]

Other publications disclose results of using the herpesvirus vector. Specifically, there has been proposed a method in which malignant cells of hematopoietic cell lines are transformed to induce expression of foreign gene substances in the cells (for example, see Patent Publication 2). However, the publication merely describes herpes simplex virus type 1, and does not disclose anything about producing methods of HHV-6 or HHV-7 vectors, or side effects of the gene therapy.

[Patent Citation 1] European Patent No. 176170

[Patent Citation 2] Japanese Laid-Open PCT Publication No. 11-513565

[Non-Patent Citation 1] Clin. Microbiol. Rev., July, 1997, Vol. 10, No. 3, p.521-567

[Non-Patent Citation 2] J. Virol. Meth., September 2002, Vol. 105, No. 2, p.331-341

[Non-Patent Citation 3] Yuji Isegawa et al., J. Virol., October 1999, Vol. 73, No. 10, p.8053-8063

[Non-Patent Citation 4] A. George Megaw et al., Virology, 1998, Vol. 244, p.119-132

[DISCLOSURE OF THE INVENTION]

[TECHNICAL PROBLEM]

[0022]

An object of the present invention is to provide a virus vector that (i) allows for insertion of an exogenous nucleotide sequence, (ii) can easily transfect a host cell of mammals, (iii) allows a gene encoded by the exogenous nucleotide sequence to be expressed in the host cell, (iv) has a low risk of pathogenicity, and therefore (v) is suitable for gene therapy of mammals.

[0023]

Another object of the present invention is to provide a virus vector producing method for easily and safely producing a virus vector that (i) allows for insertion of an exogenous nucleotide sequence, (ii) can easily transfect a host cell of mammals, (iii) allows a gene encoded by the exogenous nucleotide sequence to be expressed in the host cell, (iv) has a low risk of pathogenicity, and therefore (v) is suitable for gene therapy of mammals.

[0024]

Another object of the present invention is to provide a host cell transforming method for transforming a host cell with a virus vector that (i) easily allows for transfection of a mammalian host cell with an exogenous nucleotide sequence, (ii) allows a gene encoded by the exogenous nucleotide sequence to be expressed in the host cell, (iii) has a low risk of pathogenicity, and therefore (iv) is suitable for gene therapy of mammals.

[0025]

Another object of the present invention is to provide a transformed host cell that (i) is transformed by a virus vector with the insertion of an exogenous nucleotide sequence, (ii) allows a gene encoded by the exogenous nucleotide sequence to be expressed in the host cell, (iii) has a low risk of pathogenicity, and therefore (iv) can

suitably be used for gene therapy and cell therapy.

[0026]

Another object of the present invention is to provide a gene therapy method for mammals using a virus vector that (i) easily allows for transfection of a mammalian host cell with an exogenous nucleotide sequence, (ii) allows a gene encoded by the exogenous nucleotide sequence to be expressed in the host cell, and (iii) has a low risk of pathogenicity.

[0027]

Another object of the present invention is to develop a gene therapy method, a recombinant virus, and a recombinant virus vector with the use of viruses which do not pose problems of conventionally used viruses, including poor gene introduction efficiency, instable gene expression, and a potential risk of causing cancer.

[TECHNICAL SOLUTION]

[0028]

The inventors of the present invention diligently worked to solve the foregoing problems by contemplating that HHV-6 or HHV-7, which produces fairly mild symptoms and latently infects nearly 100% of healthy adult individuals may be suitably used as a virus vector for gene therapy.

[0029]

Specifically, in order to make a recombinant virus, the inventors of the present invention conducted trial and error experiments in an effort to find dispensable regions that can be replaced with drug resistant genes, as will be described later in Examples.

[0030]

As a result, the inventors of the present invention found a gene cluster that is non-essential and therefore dispensable for the replication and latency of human herpesvirus 6 (HHV-6) and human herpesvirus 7 (HHV-7), as will be described later in Examples.

[0031]

Based on this finding, the inventors of the present invention accomplished the present invention by finding that insertion of an exogenous nucleotide sequence in a specific region of HHV-6 or HHV-7 does not impair functions of HHV-6 or HHV-7 as a virus vector, thereby enabling production of a recombinant virus and recombinant virus vector originating in HHV-6 and HHV-7, which is very difficult with conventional techniques.

[0032]

Specifically, a recombinant virus vector of the present invention originates in HHV-6 and includes an exogenous nucleotide sequence in a portion corresponding to at least one region selected from the group consisting of U2, U3, U4, U5, U6, U7, and U8 regions of HHV-6.

[0033]

It is preferable that the portion exist between nucleotide numbers 10216 and 16547 of a HHV-6 DNA sequence as represented by SEQ ID NO: 1. It is also preferable that the recombinant virus vector comprises H6R28 virus or H6R28 virus.

[0034]

A recombinant virus vector of the present invention may originate in HHV-7 and include an exogenous nucleotide sequence in a portion corresponding to at least one region selected from the group consisting of U2, U3, U4, U7, and U8 regions of HHV-7.

[0035]
It is preferable that the portion exists between nucleotide numbers 10558 and 18483 of a HHV-7 DNA sequence as represented by SEQ ID NO: 2. It is also preferable that the recombinant virus vector comprises H7R28 virus.

[0036]
The exogenous nucleotide sequence may be a DNA sequence and/or RNA sequence.

[0037]
The exogenous nucleotide sequence may encode at least one kind of substance selected from the group consisting of a bacterial artificial chromosome (BAC), cytokine gene, ribozyme, interference RNA, immunological co-stimulator molecule, signal transduction molecule, enzyme, and chemical attractant.

[0038]
Further, the exogenous nucleotide sequence may be used for gene therapy of mammals. The exogenous nucleotide sequence may include a nucleotide sequence that encodes a marker gene.

[0039]
A producing method of a recombinant virus of the present invention originates in HHV-6, and the method includes the step of inserting an exogenous nucleotide sequence in a portion corresponding to at least one region selected from the group consisting of U2, U3, U4, U5, U6, U7, and U8 regions of HHV-6.

[0040]
It is preferable that, in the step of inserting an exogenous nucleotide sequence, the exogenous nucleotide sequence be inserted between nucleotide numbers 10216 and 16547 of a HHV-6 DNA sequence as represented by SEQ ID NO:

1.

[0041]

In the step of inserting an exogenous nucleotide sequence, homologous recombination may be carried out between a HHV-6 DNA sequence and a DNA sequence that is amplified with a primer set of a sequence represented by SEQ ID NO: 3-4 and a sequence represented by SEQ ID NO: 5-6.

[0042]

A producing method of a recombinant virus vector of the present invention may originate in HHV-7, and the method may include the step of inserting an exogenous nucleotide sequence in a portion corresponding to at least one region selected from the group consisting of U2, U3, U4, U7, and U8 regions of HHV-7.

[0043]

In the step of inserting an exogenous nucleotide sequence, the exogenous nucleotide sequence may be inserted between nucleotide numbers 10558 and 18483 of a HHV-7 DNA sequence as represented by SEQ ID NO: 2.

[0044]

In the step of inserting an exogenous nucleotide sequence, the exogenous nucleotide sequence may be inserted inside a normal cell and/or an umbilical cord blood cell.

[0045]

A transforming method of a host cell of the present invention transforms a host cell of a mammal with the recombinant virus vector of the invention, and the method includes the step of transforming, with the recombinant virus vector, a host cell of at least one kind of mammal selected from the group consisting of a human, a non-human primate, and a host that is open to HHV-6 or

HHV-7 infection.

[0046]

In the step of transforming a host cell, the method may transform, with the recombinant virus vector, at least one kind of host cell selected from the group consisting of a T cell, macrophage, glial cell, peripheral-blood mononuclear cell, blood stem cell, liver cell, fibroblast, and natural killer cell.

[0047]

A transformed host cell of the present invention is obtained by the transforming method of the invention. A transformed host cell of the present invention may be used for gene therapy of mammals.

[0048]

Further, the gene therapy may be for preventing human immunodeficiency virus (HIV) infection of a compromised cell caused by HIV, and/or for immunotherapy of cancer. The host cell may be derived from a mammal of the kind subjected to the gene therapy.

[0049]

A gene therapy method of the present invention is for non-human mammals, and the method includes the step of administering the transformed cell of the invention.

[0050]

A gene therapy method of the present invention is for non-human mammals, and the method may include the step of transforming, with a recombinant virus vector of claim 1 or 4, a host cell of a mammal in vivo at a multiplicity of infection (MOI) of 0.01 to 20.

[0051]

A gene therapy method of the present invention may include the step of expressing a gene encoded by the

exogenous nucleotide sequence included in the recombinant virus vector.

[ADVANTAGEOUS EFFECTS]

[0052]

As described below, a virus vector of the present invention (i) allows for insertion of an exogenous nucleotide sequence, (ii) can easily transfect a host cell of mammals, (iii) allows a gene encoded by the exogenous nucleotide sequence to be expressed in the host cell, (iv) has a low risk of pathogenicity, and therefore (v) is suitable for gene therapy of mammals.

[0053]

A producing method of a virus vector of the present invention is for easily producing a virus vector that (i) allows for insertion of an exogenous nucleotide sequence, (ii) can easily transfect a host cell of mammals, (iii) allows a gene encoded by the exogenous nucleotide sequence to be expressed in the host cell, (iv) has a low risk of pathogenicity, and therefore (v) is suitable for gene therapy of mammals.

[0054]

A transforming method of a host cell of the present invention is a method for transforming a host cell with a virus vector that (i) easily allows for transfection of a mammalian host cell with an exogenous nucleotide sequence, (ii) allows a gene encoded by the exogenous nucleotide sequence to be expressed in the host cell, (iii) has a low risk of pathogenicity, and (v) therefore is suitable for gene therapy of mammals.

[0055]

A transformed host cell of the present invention (i) is

transformed with a virus vector with the insertion of an exogenous nucleotide sequence, (ii) allows a gene encoded by the exogenous nucleotide sequence to be expressed in the host cell, (iii) has a low risk of pathogenicity, and therefore (iv) can suitably be used for gene therapy.

[0056]

A gene therapy method of the present invention is a gene therapy method for mammals using a virus vector that (i) easily allows for transfection of a mammalian host cell with an exogenous nucleotide sequence, (ii) allows a gene encoded by the exogenous nucleotide sequence to be expressed in the host cell, and (iii) has a low risk of pathogenicity.

[0057]

Virus vectors of the present invention can be used for AIDS treatment by taking advantage of the fact that both HHV-6 and HHV-7 infect the CD4 positive T cells as does HHV. In this case, it is preferable that virus vectors of the present invention include anti-HIV genes such as ribozyme and interference RNA.

[0058]

Further, virus vectors of the present invention can be used for AIDS treatment by taking advantage of the fact that HHV-6 can latently infect macrophage as does HIV. In this case, it is also preferable that virus vectors of the present invention include anti-HIV genes such as ribozyme and interference RNA.

[0059]

Further, virus vectors of the present invention can be used to introduce cytokine to the CD4 positive T cells, macrophage, natural killer cells, lymphokine activated killer (LAK) cells, and the like by taking advantage of the

fact that both HHV-6 and HHV-7 infect the immunocompetent cells of these cells. Thus, virus vectors of the present invention can be used for the immunotherapy of cancer.

[0060]

Further, virus vectors of the present invention are also applicable to anti-tumor treatment. In this case, pancreatic cancer cells are infected with HHV-6 to kill cancer cells, by taking advantage of the fact that HHV-6 can enter the cells by binding to CD46 molecules serving as receptors, which are abundantly expressed in pancreatic cancer cells or other refractory digestive system tumors.

[BEST MODE FOR CARRYING OUT THE INVENTION]

[0061]

The present invention is now described in detail in the embodiment below.

[0062]

<Definitions>

As used herein, HHV-6 refers to variants A and B of human herpesvirus 6.

[0063]

As used herein, HHV-7 refers to human herpesvirus 7.

[0064]

As used herein, a recombinant virus and a recombinant virus vector refer to a virus or a virus vector that is prepared by incorporating foreign genes in viral genes, and that causes either one of productive infection, latent infection/reactivation, and abortive infection when used to infect a host cell.

[0065]

As used herein, a dispensable region refers to a viral gene region, the lack of which does not lead to a complete loss of the proliferating ability of the virus.

[0066]

As used herein, an exogenous nucleotide sequence refers to a nucleic acid sequence other than those found in naturally occurring viral genes.

[0067]

As used herein, latent infection refers to a situation where production of infectious virus is suspended with the viral genes retained in the virus.

[0068]

As used herein, infection refers to entry of virus into a cell, and it includes productive infection, latent infection, and abortive infection.

[0069]

As used herein, abortive infection refers to a situation where a virus that has entered a cell does not actively retain viral genes while no infectious virus is produced.

[0070]

As used herein, gene therapy refers to therapy in which a cell is transformed by transfecting the cell with foreign genes. In a narrow sense, it includes cell therapy in which a cell that has been transformed ex vivo by gene transfection is returned to the living organism, and virus therapy in which a cell is infected with infectious virus in vivo in order to facilitate modification of the host cell by a resulting virus.

[0071]

<HHV-6-Derived Recombinant Virus and Recombinant Virus Vector>

A recombinant virus and recombinant virus vector of the

present invention are derived from HHV-6 and include an exogenous nucleotide sequence in a portion corresponding to at least one region selected from the group consisting of U2, U3, U4, U5, U6, U7, and U8 regions of HHV-6.

[0072]

The U2 region of HHV-6 is an open reading frame (ORF) encoded by nucleotide number 10768 (start) to nucleotide number 9467 (end) of HHV-6 as represented by SEQ ID NO: 1, and it belongs to the US22 gene family of HCMV.

[0073]

The U3 region of HHV-6 is an open reading frame (ORF) encoded by nucleotide number 12051 (start) to nucleotide number 10891 (end) of HHV-6 as represented by SEQ ID NO: 1, and it belongs to the US22 gene family of HCMV.

[0074]

The U4 region of HHV-6 is an open reading frame (ORF) encoded by nucleotide number 13883 (start) to nucleotide number 12276 (end) of HHV-6 as represented by SEQ ID NO: 1, and it has unknown functions.

[0075]

The U5 region of HHV-6 is an open reading frame (ORF) encoded by nucleotide number 15333 (start) to nucleotide number 14002 (end) of HHV-6 as represented by SEQ ID NO: 1, and it has unknown functions.

[0076]

The U6 region of HHV-6 is an open reading frame (ORF) encoded by nucleotide number 15395 (start) to nucleotide number 15652 (end) of HHV-6 as represented by SEQ ID NO: 1, and it has unknown functions.

[0077]

The U7 region of HHV-6 is an open reading frame (ORF) encoded by nucleotide number 16802 (start) to nucleotide

number 15678 (end) of HHV-6 as represented by SEQ ID NO: 1, and it belongs to the US22 gene family of HCMV.

[0078]

The U8 region of HHV-6 is an open reading frame (ORF) encoded by nucleotide number 18041 (start) to nucleotide number 16806 (end) of HHV-6 as represented by SEQ ID NO: 1, and it belongs to the US22 gene family of HCMV.

[0079]

The foregoing portions of HHV-6 may exist between nucleotide number 9467 and nucleotide number 18041 of the DNA sequence of HHV-6 as represented by SEQ ID NO: 1. This is because nucleotide number 9467 to nucleotide number 18041 contains U2 region to U8 region, which were found to be dispensable as will be described later in Examples.

[0080]

The foregoing portions of HHV-6 may exist between nucleotide number 10216 and nucleotide number 16547 of the DNA sequence of HHV-6 as represented by SEQ ID NO: 1. This is because nucleotide number 10216 to nucleotide number 16547 were experimentally confirmed to be dispensable as will be described later in Examples.

[0081]

A desirable exogenous nucleotide can easily be inserted in these portions in the manner described below. First, the HHV-6 DNA is cut at restriction enzyme cutting sites in these portions under appropriate conditions, using commercially available restriction enzymes. Then, the HHV-6 DNA is ligated under appropriate conditions to an exogenous nucleotide having complementary ends, using a commercially available ligase.

[0082]

As to functions of US22 family genes, some information is available for human cytomegalovirus or mouse cytomegalovirus that belongs to β-herpes virus as does HHV-6 or HHV-7. However, no information is available for HHV-6 and HHV-7. The present invention, for the first time, analyzed functions of US22 family genes of HHV-6 and HHV-7 concerning their proliferation and latency.

[0083]

The US22 family genes of HHV-6 and HHV-7 are merely classified according to a virtual motif on amino acid sequences whose functions are yet to be determined. As such, the fact that the genes belong to this family does not necessarily mean that their functions can be predicted. Further, there is no strong amino acid homology between homologous proteins of the HHV-6, HHV-7, and cytomegalovirus.

[0084]

<HHV-7-Derived Recombinant Virus and Recombinant Virus Vector>

A recombinant virus and recombinant virus vector of the present invention may be derived from HHV-7 and may include an exogenous nucleotide sequence in a portion corresponding to at least one region selected from the group consisting of U2, U3, U4, U7, and U8 regions of HHV-7. Note that, although no experiment was carried out with respect to HHV-7, it is conceivable that HHV-7 is usable as virus vector as is the HHV-6, on the ground of the high homology between HHV-7 and HHV-6.

[0085]

The U2 region of HHV-7 is an open reading frame (ORF) encoded by nucleotide number 11637 (start) to nucleotide number 10558 (end) of HHV-7 as represented by SEQ ID

NO: 2, and it shares a common motif with the US22 gene family of HCMV.

[0086]

The U3 region of HHV-7 is an open reading frame (ORF) encoded by nucleotide number 12953 (start) to nucleotide number 11799 (end) of HHV-7 as represented by SEQ ID NO: 2, and it shares a common motif with the US22 gene family of HCMV.

[0087]

The U7 region of HHV-7 is an open reading frame (ORF) encoded by nucleotide 14603 (start) to nucleotide number 12975 (end) of HHV-7 as represented by SEQ ID NO: 2, and it is associated with the U4 region of exon 2.

[0088]

Exon 1 (also known as U5) in the U7 region of HHV-7 is an open reading frame (ORF) encoded by nucleotide number 17324 (start) to nucleotide number 16348 (end) of HHV-7 as represented by SEQ ID NO: 2, and it shares a common motif with the US22 gene family of HCMV.

[0089]

Exon 2 in the U7 region of HHV-7 is an open reading frame (ORF) encoded by nucleotide number 16266 (start) to nucleotide number 14628 (end) of HHV-7 as represented by SEQ ID NO: 2, and it is associated with U4.

[0090]

The foregoing portions of HHV-7 may exist between nucleotide number 10558 and nucleotide number 18483 of the DNA sequence of HHV-7 as represented by SEQ ID NO: 2. This is because nucleotide number 10558 to nucleotide number 18483 contains U2, U3, U4, U7, and U8 regions of HHV-7, which correspond to U2 through U8

of HHV-6, and which were found to be dispensable as will be described later in Examples.

[0091]

A desirable exogenous nucleotide can easily be inserted in these portions in the manner described below. First, the HHV-7 DNA is cut at restriction enzyme cutting sites in these portions under appropriate conditions, using commercially available restriction enzymes. Then, the HHV-7 DNA is ligated under appropriate conditions to an exogenous nucleotide having complementary ends, using a commercially available ligase.

[0092]

<Exogenous Nucleotide Sequence>

The exogenous nucleotide sequence may be a DNA sequence and/or an RNA sequence. The DNA sequence may be a genomic DNA sequence or cDNA sequence.

[0093]

Further, the exogenous nucleotide sequence may be a nucleotide sequence that encodes one or more substances selected from the group consisting of a bacterial artificial chromosome (BAC), a cytokine gene, a ribozyme, interference RNA, immunological co-stimulator molecule, and a chemical attractant.

[0094]

Further, the exogenous nucleotide sequence may be a sequence used for gene therapy of mammals. Further, the exogenous nucleotide sequence may be a nucleotide sequence that encodes an immunoregulatory protein useful for a tumor treatment and/or immune treatment of mammals.

[0095]

Further, the exogenous nucleotide sequence may include

a nucleotide sequence that encodes a maker gene. The marker gene may be an antibiotic-resistant gene.

[0096]

<Producing Method of Recombinant Virus and Recombinant Virus Vector>

A producing method of a recombinant virus and recombinant virus vector of the present invention is for producing a recombinant virus and recombinant virus vector derived from HHV-6, the method including the step of inserting an exogenous nucleotide sequence in a portion corresponding to at least one region selected from the group consisting of U2, U3, U5, U6, U7, and U8 regions of HHV-6.

[0097]

The step of inserting an exogenous nucleotide sequence preferably includes the step of cutting the HHV-6 DNA under appropriate conditions at restriction enzyme cutting sites in the foregoing portion, using a commercially available restriction enzyme, and the step of ligating the HHV-6 DNA under appropriate conditions with an exogenous nucleotide having complementary ends, using a commercially available ligase. In this manner, with the commercially available restriction enzyme and ligase, a desirable exogenous nucleotide can easily be inserted in the foregoing portions.

[0098]

The step of inserting an exogenous nucleotide sequence may include the step of inserting an exogenous nucleotide sequence between nucleotide number 9467 and nucleotide number 18041 of the DNA sequence of HHV-6 as represented by SEQ ID NO: 1. This is because nucleotide number 9467 to nucleotide number 18041 contains U2

region to U8 region of HHV-6, which were found to be dispensable as will be described later in Examples.

[0099]

Further, the step of inserting an exogenous nucleotide sequence may include the step of inserting an exogenous nucleotide sequence between nucleotide number 10216 and nucleotide number 16547 of the DNA sequence of HHV-6 as represented by SEQ ID NO: 1. This is because nucleotide number 10216 to nucleotide number 16547 were experimentally confirmed to be dispensable as will be described later in Examples.

[0100]

Further, the step of inserting an exogenous nucleotide sequence may include the step of performing homologous recombination between a DNA sequence of HHV-6 and a DNA sequence amplified with a combination of primers having the sequences of SEQ ID NOs: 3 and 4 and primers having the sequences of SEQ ID NOs: 5 and 6.

[0101]

[TABLE1]

Primer Used for PCR

PRIMER	SEQ ID NO:	PRIMER SEQUENCE
U2 Xba I	3	5'- GCTCTAGACTGCCAOGTGAGCGAAAGCATAOAG -3'
U2 AflII	4	5'- TTAGTTAAGTCATCGGGGTCAGTATCTTGGCAG -3'
U8 Bam HI	5	5'- GCGGATCCGAGTTAATGCATAOATGGGAGGCOAGG -3'
U8 Eco RI	6	5'- CGGAATTCCTGTGTACCGTCATGGCTTGT -3'
U2R1	7	5'- GACATGCCTACGCGGGCTCACGCAG -3'
U2R2	8	5'- GGAGCGGTGTAGAAAGGGCAACG -3'
U8F1	9	5'- GTAGTAACATCATCACATGCGCGGAG -3'
U8F2	10	5'- GGGAGTATTCAGATTCGTTACAGAG -3'
EGFPprim	11	5'- GACTCAGATCTCGAGCTCAAGCTTCG -3'
PAGprim	12	5'- CCTTGTACAGCGGGGTCGGGTTCAC -3'
U3F	13	5'- ATGGAAACGACAAAAGAGCGGGGTG -3'
U3R	14	5'- GTATCGAACTACGTCGTGCACGGC -3'
U4F	15	5'- GGGCAGGTTGACCGCAGAGGATCTG -3'
U4R	16	5'- GTCCGGTGCTCCTGCAACGTGATGTC -3'
U5F	17	5'- GAAGTGGCAGTCCATCAGTAAACTGGC -3'
U5R	18	5'- CCATTCAGTTCGCCGGCACAGTCC -3'
U6F	19	5'- GCCAAATCTAGCTGCTGAGGTTCCC -3'
U6R	20	5'- CTCTTTGCGGTGGGTCTGGACGAAG -3'
U7F	21	5'- GAGGGCGGAAAGCGAGTCTTGTGTG -3'
U7R	22	5'- CATCCTGAAGAATTGTCTTTCGGG -3'
EGFP R1	23	5'- CGTAGCGTTCGGGGATGGCGGAGTTG -3'
EGFP R2	24	5'- CCTTCAGCTCGATGCGGTTCACCAGG -3'
RL-1	25	5'-GTTATGAGTATTTCTTCCAGGGTATCGAGGGTGGGTA GTCCCCACGTTTGTAGATTTTTTTTTTTTTTTTT- 3'
N1	26	5'- GCTGGGTAGTCCCCACGTTTGTAGA -3'
N2	27	5'- CTTATGAGTATTTCTTCCAGGGTATCGAG -3'

Annealing site and direction of primer are shown in Figures 1 and 6A

[0102]

A producing method of a recombinant virus and recombinant virus vector of the present invention is for producing a recombinant virus and recombinant virus vector derived from HHV-7, the method including the step of inserting an exogenous nucleotide sequence in a portion corresponding to at least one region selected from the group consisting of U2, U3, U4, U7, and U8 regions of HHV-7.

[0103]

The step of inserting an exogenous nucleotide sequence preferably includes the step of cutting the HHV-7 DNA under appropriate conditions at restriction enzyme cutting sites in the foregoing portion, using a commercially available restriction enzyme, and the step of ligating the HHV-7 DNA under appropriate conditions with an exogenous nucleotide having complementary ends, using a commercially available ligase. In this manner, with the commercially available restriction enzyme and ligase, a desirable exogenous nucleotide can easily be inserted in the foregoing portions.

[0104]

The step of inserting an exogenous nucleotide sequence may include the step of inserting an exogenous nucleotide sequence between nucleotide number 10558 and nucleotide number 18483 of the DNA sequence of HHV-7 as represented by SEQ ID NO: 2. This is because nucleotide number 10558 to nucleotide number 18483 contains U2, U3, U4, U7, and U8 regions of HHV-6, which were found to be dispensable as will be described later in Examples.

[0105]

Note that, the step of inserting an exogenous nucleotide may include the step of inserting an exogenous nucleotide sequence inside a normal cell and/or a normal umbilical cord blood cell. A drawback of the adenovirus conventionally used to construct a recombinant virus vector is that construction of recombinant virus is difficult unless it is performed inside HEK293 cell lines derived from kidney cancer cells. An advantage of a recombinant virus vector of the present invention, on the other hand, is that it can be constructed inside a normal cell, or more preferably inside a normal umbilical cord blood cell.

[0106]

<Transformation Method of Host Cell>

A method for transforming a host cell of the present invention is for transforming a host cell of mammals with use of the recombinant virus and recombinant virus vector, and the method includes the step of transforming a host cell with the recombinant virus and recombinant virus vector at a multiplicity of infection (MOI) of 0.01 to 20.

[0107]

The transformation step may include the step of transforming, with the recombinant virus and recombinant virus vector, a host cell derived from one or more kinds of mammals selected from the group consisting of a human, a non-human primate, mouse.

[0108]

Further, the transformation step may include the step of transforming, with the recombinant virus and recombinant virus vector, at least one kind of a host cell selected from the group consisting of a T cell, macrophage,

glial cell, peripheral-blood mononuclear cell, blood stem cell, liver cell, fibroblast, and natural killer cell. Conventionally, these cells had the problem of transfection efficiency and expression when used with conventional vectors. With a recombinant virus vector of the present invention, foreign genes can be introduced into these cells and expressed therein.

[0109]

The transformation step may be performed either ex vivo or in vivo.

[0110]

<Transformed Host Cells>

A transformed host cell of the present invention is obtained by the foregoing method of transforming a host cell.

[0111]

A transformed host cell of the present invention may be used in gene therapy methods of mammals. The gene therapy for which a transformed host cell of the present invention is used may be gene therapy (i) for preventing human immunodeficiency virus (HIV) infection in a compromised cell caused by HIV, and/or (ii) for immunotherapy of cancer.

[0112]

Further, the host cell may be derived from a mammal of the kind subjected to the gene therapy.

[0113]

<Gene Therapy Method>

A gene therapy method of the present invention is for non-human mammals, and it includes the step of administering the transformed cell into such mammals.

[0114]

A gene therapy method of the present invention may be used not only for gene therapy but also for virus therapy and cell therapy as well. The cell therapy refers to a method in which a cell that has been transformed by gene transfection is administered to a patient. The virus therapy refers to a method in which a patient is administered with a virus that is infectious and is intended to multiply inside the human body.

[0115]

A gene therapy method of the present invention is for non-human mammals, and includes the step of transforming, with use of the recombinant virus and recombinant virus vector, a host cell inside the body of the mammal at a multiplicity of infection (MOI) of 0.01 to 20.

[0116]

A gene therapy method of the present invention may include the step of expressing a gene encoded by an exogenous nucleotide sequence included in the recombinant virus and recombinant virus vector.

[0117]

The following will describe the present invention in more detail based on Examples. It should be noted however that the present invention is not limited in any way by the following description.

[0118]

The following Examples were carried out with samples obtained with the informed consent of the blood donors who participated in the study.

[0119]

<Construction of HHV-6 Recombinant Virus Vector>

In order to construct recombinant virus H628R, a U3-U7

gene cluster of human herpesvirus 6 (HHV-6) was replaced with EGFP-puro, a gene cassette containing the gene for enhanced green fluorescent protein (EGFP) under control of the human cytomegalovirus major immediate-early enhancer-promoter (MIEP) and the puromycin resistance gene under control of the simian virus 40 (SV40) early promoter. To insert the EGFP-puro cassette into the HHV-6 genome by homologous recombination, 1-kb segments of viral genome were inserted into each end of the cassette (Figure 1).

[0120]

The following gene clusters were examined: the DR2-DR7 genes, which are duplicated in the viral genome; U95, the positional homologue of the murine cytomegalovirus (MCMV) immediate-early (IE) 2 gene, which is known to be dispensable for viral replication; and the U3-U7 genes. Of these, it was found that replacement of the U3-U7 genes with EGFP-puro resulted in a successfully replicating virus.

[0121]

Figure 1 schematizes a structure of the H6R28 genome.

[0122]

At the top is a map of the HHV-6B HST genome, with the region U1 to U9 expanded below.

[0123]

Shaded arrows in the middle show the U3-U7 open reading frames replaced by the EGFP-puro cassette (aligned in the region of from 10315 to 16302). The position setting of PCR primer used for duplication of U2 and U8 DNA fragments (U2 XBaI, U2 Af1II, U8 BamHI and U8 EcoRI), and the priver for prooving the recombinant virus are illustrated. Primer sequence is shown in Table

1.

[0124]

A diagram at the bottom shows EGFP-puro cassette pU2-U8 EGFP-puro. White square indicates main early promoter gene of the human cytomegalovirus derived from EGFP gene and pEGFP-C1(Nucleotide number 8 to 1640: CLONTECH).

[0125]

Multiple cloning sites of pEGFP-C1 including PstI were deleted. The puromycin-N-acetyl-transferase gene (pac) and SV40 early promoter gene were derived from pPUR (nucleotide numbers 408 to 1392) (Clontech).

[0126]

The annealing site of the primer used is shown by the small black arrow. The recognition sites (PstI, AflII and Bam HI) of restriction enzyme used are shown likewise. Dotted arrow indicates the size of amplified or cut sites.

[0127]

In order to construct the recombinant virus vector, the U2 gene was amplified by PCR with primers U2 XbaI and U2 AflII, and the U8 gene was amplified with U8 BamHI and U8 EcoRI. After a restriction enzyme digestion, the digested products were inserted into each end of pEGFP-puro (pU2-U8 EGFP-puro of Figure 1).

[0128]

The cloned plasmid pU2-U8 EGFP-puro was introduced into phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells by using a Nucleofector™ electroporator (Amaxa Biosystems, Germany) according to the manufacturer's recommended protocol.

[0129]

Briefly, 5×10^6 cells were mixed with 5 μg of the plasmid

and 100 µl of Nucleofector™ solution for T cells, and electroporation was performed with the Nucleofector™ using the program U-14. [0130]

Alternatively, a conventional electroporation method was used. In this case, 1×10^7 cells were mixed with 50 µg of the plasmid suspended in 500 µl of K-phosphate-buffered saline (30.8 mM NaCl, 120.7 mM KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4, and 25 mM $MgCl_2$), and the mixture was placed in an electroporation cuvette (Gene Pulser cuvette, 0.4-cm diameter; Bio-Rad).

[0131]

Electroporation was performed with a Gene Pulser II electroporation system (Bio-Rad) with maximum resistance, voltage at 300 V, and capacitance at 960 µF. After 6 hours, the cells were infected with HHV-6 variant B of the HST strain at a multiplicity of infection (MOI) of 0.5 using the centrifuge method.

[0132]

Cells were cultured for 3 days in RPMI 1640 supplemented with 10% fetal bovine serum and frozen as a virus stock. To enrich for the recombinant virus, PHA-stimulated umbilical cord blood mononuclear cells (CBMCs) were infected with the virus stock and cultured for 1 day, treated with 7.5 µg of puromycin/ml for 1 day, washed with the medium, and cultured with CBMCs for 3 days. The infected cells were then frozen as a new virus stock.

[0133]

This selection procedure was repeated five times, and the recombinant virus (H6R28) was subsequently cloned by limiting dilution using CBMCs cultured in 96-well plates, and the clone was isolated. An informed consent was

obtained from a blood donor on participation in the study.

[0134]

<Construction Confirmation of HHV-6 Recombinant Virus Vector>

[0135]

To confirm the insertion of the EGFP-puro cassette into the expected region, viral DNA was amplified by double-nested PCR with KOD Plus DNA polymerase (TOYOBO, Otsu, Japan) using primers against regions outside the homologous hinge regions: U2R2-U8F2 (Figure 1).

[0136]

As a result, an amplified product of approximately 8.5 kb was observed in the wild-type (wt) virus, and approximately 5.0 kb was observed in three clones of H6R28 (Representative examples are shown in Figure 2(A))[0137]

The treatment of the amplified products with the restriction enzymes of Figure 1 gave rise to the expected bands in both wt virus and H6R28 (Figure 2(A)). The amplified products were confirmed by partial sequencing (data not shown). A product of 8.5 kb was not observed in the recombinant virus, meaning that the recombinant virus is not infected by the WT virus.

[0138]

An insertion site of EGFP-puro were studied likewise by using a primer homological with EGFP-puro, and an expected PCR product was observed (Figure 2 (B)). In order to address the possibility of the ectopic expression of U3-U7 genes, the inventor attempted amplification of each gene from H6R28 with the use of PCR. However, the amplified product was not obtained (Figure 2(c)).

[0139]
Figure 2 is a electrophoretic picture showing PCR amplification of viral DNAs of the wild-type (wt) virus and H6R28 virus.

[0140]
Note that, in Figure 2(A), the viral DNAs from the Wt (lanes 1 and 3) and H6R28 (lanes 2, 4, 5, and 6) are amplified by double-nested PCR, using pairs of primers U2R2-U8F2, and U2R1-U8F1.

[0141]
The amplified fragments are digested with PstI (lanes 3 and 4), or BamHI (lane 6). Non-digested fragments are separated with 0.6% agarose gel (lanes 1 and 2), the digested fragments are separated with 1.0 gel (lane 3 to lane 6).

[0142]
Figure 1 illustrates locations of fragments from Wt virus, and fragments from H6R28. White arrow is placed nearby an expected site and the size of the Wt virus, and black arrow is placed nearby an expected site and the size of the fragments from H6R28.

[0143]
Figure 2(B) illustrates DNAs from the H6R28 are mixed with primer U2R1-EGFPp rim (lane 1) or primer U8F1-PACp rim (lane 2), and are amplified by PCR. The amplified fragments are isolated by using 1.0% agarose gel. The arrow illustrates an expected size of the fragments. The size of U2R1-EGFPp rim is 1582bp, and that of PACp rim-U8R1 is 1760bp.

[0144]
Figure 2(C) indicates PCR amplification of deleted ORF.

[0145]

Viral DNAs from wt (lanes 1 to 5) and H6R28 (lanes 6 to 10) are subject to PCR amplification using pair of primers U3F1-U3R1(lanes 1 and 6), or U7F1-U7R1 (lanes 5 and 10).

[0146]

The amplified fragments are isolated by using 1.0 % agarose gel. The sizes of U3F-U3R, U4F-U4R, U5F-U5R, U6F-U6R, and U7F-U7R were 1161 bp, 1338 bp, 1275 bp, 171 bp, and 1094 bp, respectively. The lane M indiccates 1Kbp Ladder Plus (Invitrogen).

[0147]

The inventors of the present invention produced three independent isolates of H6R28 by three individual electroporations and examined the replication speed in CBMCs. Virus titration was performed using CBMCs as described above. CBMCs were infected at a MOI of 0.05, and the three H6R28 clones and the wt virus showed similar levels of viral spreading (Figure 3(A)) and virus production (Figure 3(B)) over time.

[0148]

Figure 3 a graph representing productive infection of H6R28.

[0149]

Figure 3(A) represents kinetics of the increase in cells infected with wt virus and H6R28. CBMCs were infected with wt virus and three independent clones of H6R28 at an MOI of 0.05 (50% tissue culture infectious doses (TCID50)/cell), and the percentages of cells reacting with a mixture of monoclonal antibodies to gB and gH were determined by IFA staining using monoclonal antibodies. The percentages of cells infected with wt virus (open circle), H6R28 clone 1 (solid triangle), clone 2 (solid circle), and clone 3 (solid square) are shown. Data shown

are mean values of results for three replicate cultures.

[0150]

Figure 3(B) represents growth curves for wt virus and H6R28.

[0151]

CBMCs were infected as described above, and infected cells were harvested at the indicated times and frozen at -80°C. Progeny viruses were titrated on CBMCs using IFA staining. Virus activity was indicated as 50% TCID per milliliter. Activity of cells infected with wt virus (open square), H6R28 clone 1 (solid triangle), clone 2 (solid circle), and clone 3 (solid square) are shown. Values on day 0 represent the activity of the input viruses. Data shown are mean values of results for three replicate cultures.

[0152]

<Latent Infection Ability and Reactivation Efficiency of HHV-6 Recombinant Virus Vector>

The inventors of the present invention investigated H6R28 for its ability to establish latency and its efficiency of reactivation. To evaluate the establishment of latency, peripheral blood macrophages were infected with wt virus and H6R28 and the percentage of HHV-6 DNA(+) cells was monitored.

[0153]

Briefly, peripheral blood macrophages were cultured in RPMI 1640 supplemented with 25% horse serum on plastic plates coated with collagen (Sumitomo Bakelite Co., Ltd. Japan). The macrophages were infected with HHV-6 on day 7 and cultured for 4 to 6 weeks. The infected macrophages were detached from the plates, and the absence of viral replication was confirmed by

immunofluorescent antibody (IFA) staining using monoclonal antibodies against glycoproteins B and H.

[0154]

The cells were serially diluted (10^4 to 1 cell per tube) into sample tubes using four tubes for each dilution, and the DNA was isolated from each sample tube. Viral DNA was detected by double-nested PCR, and the numbers of HHV-6(+) DNA cells were calculated by the Reed-Muench method. According to the experiment, the percentage of the HHV-6(+) DNA cells were similar to the cultures infected by Wt virus and H6R28 virus (See Figure 4(A)).

[0155]

Figure 4(A) represents percentages of HHV-6 DNA-positive cells. The percentages of HHV-6 DNA positive cells were examined 4 and 6 weeks postinfection. The data shown are mean values and standard deviations of results for three replicate cultures of wt virus and three clones of H6R28. Open column indicates wt virus. Shaded column indicates H6R28.

[0156]

To study the reactivation efficiency, viral reactivation was induced by tetradecanoyl phorbol acetate (TPA) treatment as described above. Briefly, latently infected cells were detached from the culture dish, serially diluted, and cocultivated with an uninfected histiocyte supplying layer. Subsequently, the cells were treated with TPA (20 ng/ml) for 7 days and cocultivated with CBMCs for 7 days.

[0157]

The efficiency of the viral reactivation was calculated by the Reed-Muench method (Figure 4(B)). it was concluded that the establishment of latency and the reactivation process were not impaired by the deletion of the U3-U7

genes.

[0158]

Figure 4(B) represents percentages of reactivation positive cells. Viral reactivation was induced, and the percentages of reactivation-positive cells were calculated. The data shown are mean values and standard deviations of results for three replicate cultures of wt virus and three clones of H6R28. Open column indicates wt virus. Shaded column indicates H6R28.

[0159]

Interestingly, during HHV-6 latency, the inventors of the present invention failed to detect the expression of EGFP that was driven by the HCMV major immediate-early enhancer-promoter (MIEP) (Figure 5(A)). On the other hand, EGFP expression was observed in the latently infected macrophage (Figure 5(B)) transfected with the plasmid pU2-U8 EGFP-puro illustrated in Figure 1, reactivation-induced macrophages (Figure 5(C)), productively infected CBMCs and Molt-3 cells (Figures 5(D) and 5(E)), and non-infected HeLa cells (Figure 7(D)).

[0160]

Figure 5 represents fluorescence micrographs showing EGFP expression in various types of cells. In Figure 5, cultured live cells were observed under fluorescent illumination.

[0161]

Figure 5(A) shows macrophages that were latently infected with H6R28. Figure 5(B) shows latently infected macrophages that were infected by the plasmid pU2-U8 EGFP-puro shown in Figure 1 (transfection was performed as described above). Figure 5(C) shows reactivation-induced macrophages that were treated with

20 ng of TPA/ml for 7 days.

[0162]

Figure 5(D) shows CBMCs infected with H6R28. Figure 5(E) shows Molt-3 cells infected with H6R28. Figure 5(F) shows HeLa cells infected with H6R28.

[0163]

The cells were observed 4 weeks (A to C) or 2 days (E to F) postinfection. The transfected cells were observed 1 day post transfection (B).

[0164]

To investigate the gene expression from the IE1/IE2 promoter, RACE of 5'at the end of cDNA was performed. Briefly, the 5' end of the cDNA was dA tailed and annealed with an anchor primer, RL-1. The initial 10 cycles of PCR were performed with Taq polymerase (Roche Diagnostics) using the following conditions: denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C.

[0165]

PCR amplification was performed with PCR with KOD Plus DNA polymerase (TOYOBO, Otsu, Japan) using primers N1 and EGFP-R1 followed by primers N2 and EGFP-R2 (Figure 6(A)) under the following conditions: denaturation for 1 min at 94°C, annealing for 30 sec. at 65°C, and extension for 1 min at 68°C (15 cycles per amplification). The amplified products were sequenced.

[0166]

In the latent cells, transcription of the mRNA from the usual transcription start position (productive infection transcription start site [PSS]) was not detected (Figure 6(B)); however, small amounts of mRNA were transcribed from the latent infection transcription start sites (LSSs) 1

and 2 of HCMV, which are used to express the latency-associated transcripts of HCMV.

[0167]

In contrast, the PSS was used in the latently infected macrophage transfected with the plasmid pU2-U8 EGFP-puro, reactivation-induced macrophages, and the productively infected Molt-3 cells and the non-infected HeLa cells (Figure 6(B)). Since HCMV MIEP showed the latency-associated performance in the context of HHV-6 latency, it is suggested that the transcriptional control of HHV-6 latency may share some common mechanism with HCMV latency. These findings may be related to the fact that HCMV shows some similarity with HHV-6, such as the site of latency.

[0168]

Figure 6 is a schematic diagram and an electrophoretogram showing functions of the HCMV promoter in the latently infected HHV-6.

[0169]

Figure 6(A) shows HCMV IE1/IE2 promoter and PCR primers. The EGFP gene and transcription start sites are drawn to scale. The PSS of IE1/IE2 mRNA (indicated as +1) and two LSSs (LSS1 and LSS2) are shown. The locations of the PCR primers are depicted, and a schematic drawing shows the usage of the anchor primer RL-1. Primer sequences are shown in Table 1.

[0170]

Figure 6(B) represents 5' RACE amplification of the EGFP transcripts.

[0171]

Lane 1 shows RNA from 1×10^5 latently infected macrophages (Mf). Lane 2 shows 1×10^5 latently infected

macrophages that were transfected with the plasmid pU2-U8 EGFP-puro shown in Figure 1. Lane 3 shows 1×10^5 reactivation-induced macrophages. Lane 4 shows 1×10^2 productively infected Molt-3 cells. Lane 5 shows 1×10^3 abortively infected HeLa cells.

[0172]

These cells were analyzed by the 5' RACE method. The RACE method used was the same as that commonly used. The 5' end of the transcript was dA tailed and annealed with the anchor primer RL-1 (Figure 6A) and amplified first with primers N2-EGFP R2 and then with primers N1-EGFP R1. The 5' ends of the transcript initiating at PSS (up to 360 bp), LSS1 (up to 720 bp), and/or LSS2 (up to 650 bp) were detected. HaeIII-digested ΦX174 DNA fragments were used as size markers (ΦX).

[0173]

<Construction of HHV-7 Recombinant Virus Vector>

In order to construct recombinant virus H7R28, a U2-U8 gene cluster of human herpesvirus 7 (HHV-7) was replaced with EGFP-puro, a gene cassette containing the gene for enhanced green fluorescent protein (EGFP) under control of the human cytomegalovirus major immediate-early enhancer-promoter (MIEP) and the puromycin resistance gene under control of the SV40 early promoter. To insert the EGFP-puro cassette into the HHV-7 genome by homologous recombination, 1-kb segments of viral genome were inserted into each end of the cassette (Figure 7).

[0174]

The U2-U8 gene clusters were examined. It was found that replacement of the U2-U8 genes with EGFP-puro resulted in a successfully replicating virus.

[0175]
Figure 7 schematizes a structure of genome of the HHV-7 recombinant virus vector.

[0176]
In Figure 7, the U2-U8 ORF replaced by the EGFP-puro cassette (aligned in the region of from 10558 to 18483) are shown in the upper part. Additionally, the position setting of PCR primer used for duplication of U2 and U7-U8 DNA fragments (U2 SPeI, U2 BamHI, U7 BamHI, and U8 SalI), and the primer sequences are shown in the following Table 2.

[0177]
[TABLE 2]

PRIMER	SEQ ID NO:	PRIMER SEQUENCES
7U2F1	28	5'- CAGCGTTTCCTGATGTTGGAAGCCAG -3'
7U2R1	29	5'- GCATCTTACCAATGATGATGGCAAGC -3'
7U2FBam	30	5'- TTGGATCCTGATCATTTGCATGTTGCTAGTATGTCAG -3'
7U2RSpe	31	5'- GACTAGTCTCGGAATCGAAGCTAATCTGAGAGC -3'
7U8F1	32	5'- CCGATTCGTAGTTTCGAGAAGAGG -3'
7U8R1	33	5'- CTCCGTACCACAGTCTGTCTAGCTC -3'
7U8FSal	34	5'- GGGTCGACAGCCAGTTGACGTTGCTGGTTACTCAG -3'
7U8RBam	35	5'- TTGGATCCATGCCTTCTCCATATGAAGACAGCAGC -3'

Annealing site and direction of primer are shown in Figure 7.

[0178]
A diagram at the bottom shows EGFP-puro cassette pU2-U8 EGFP-puro. White square indicates the puromycin resistance gene control of EGFP gene and SV40

early promotor.

[0179]

The recognition sites (SpeI, Bg1II, Af1II, Bam HI, XhoI, Eco RI, and Sac I) of the restriction enzyme are shown like wise. The size of amplified or digested sites are indicated by the broken arrow.

[0180]

For the construct of recombinant virus vector, the U2 gene was amplified by PCR with primers 7U2F1-7U2R1, and U7-U8 genes were amplified with U2F Bam-7U2RSpe.

[0181]

The amplified products were then digested with SpeI-BamHI, and then the digested products were inserted into each end of pEGFP-puro (HHV-7 pU2-U8 EGFP-puro in Figure 7) by inserting them into digested fragments of SpeI-BglII of LIT28BAS P&E (BglII and BamHI sites are crushed).

[0182]

The cloned plasmid was transfected into phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMCs) by using a Nucleofector™ electroporator (Amaxa Biosystems, Germany) according to the manufacturer. s recommended protocol.

[0183]

Briefly, 5×10^6 cells were mixed with 5 μg of the plasmid and 100 μl of Nucleofector™ solution for T cells, and electroporation was performed with the Nucleofector™ using the program U-14. [0184]

Alternatively, a conventional electroporation method was used. In this case, 1×10^7 cells were mixed with 50 μg of the plasmid suspended in 500 μl of K-phosphate-buffered saline (30.8 mM NaCl, 120.7 mM KCl, 8.1 mM Na_2HPO_4,

1.46 mM KH_2PO4, and 25 mM $MgCl_2$), and the mixture was placed in an electroporation cuvette (Gene Pulser cuvette, 0.4-cm diameter; Bio-Rad).

[0185]

Electroporation was performed with a Gene Pulser II electroporation system (Bio-Rad) with the maximum resistance, voltage at 300 V, and capacitance at 960 μF. After 6 hours, the cells were infected with B variant of HHV-6 HST strain at a multiplicity of infection (MOI) of 0.5 using the centrifuge method.

[0186]

Cells were cultured for 3 days in RPMI 1640 supplemented with 10% fetal bovine serum and frozen as a virus strain stock. To enrich for the recombinant virus, PHA-stimulated umbilical cord blood mononuclear cells (CBMCs) were infected with the virus strain stock and cultured for 1 day, treated with 7.5 μg of puromycin/ml for 1 day, washed with the medium, and cultured with CBMCs for 3 days. The infected cells were then frozen as a new virus strain stock.

[0187]

This selection procedure was repeated five times, and the recombinant virus (H6R28) was subsequently cloned by limiting dilution using CBMCs cultured in 96-well plates. An informed consent was obtained from a blood donor on participation in the study.

[0188]

<Construction Confirmation of HHV-7 Recombinant Virus Vector>

Construction of the HHV-7 recombinant virus vector was confirmed by the method used to confirm construction of the HHV-6 recombinant virus vector. The results were the

same. To avoid redundancy, no further explanation will be made.

[0189]

<Latent infection ability and reactivation efficiency of HHV-7 recombinant virus vector>

Construction of the HHV-7 recombinant virus vector was confirmed by the method used to confirm construction of the HHV-6 recombinant virus vector. The results were the same. To avoid redundancy, no further explanation will be made.

[0190]

<Summary of Evaluation Results>

Overall, the recombinant virus revealed that the fairly large gene cluster U3-U7 was dispensable for viral replication, latency, and reactivation. Of the deleted genes, the characteristics of U4 and U6 have not been reported.

[0191]

Genes U3, U5, and U7 belong to the US22 gene family, whose members are related to the HCMV US22 gene. Every betaherpesvirus encodes several US22 family genes that encode at least one of four conserved motifs. Although the functions of most of the US22 family genes are unknown, some of them, such as the murine cytomegalovirus (MCMV) immediate-early 2 (IE2) gene and the HCMV UL36-38 genes, encode proteins with transactivating functions.

[0192]

However, MCMV IE2 is known to be dispensable for viral replication and latency and reactivation. Deletion of the US22 family genes of H6R28 showed them to have similar properties; HHV-6 U3 encodes a protein with a weak

transactivating function, and the inventors of the present invention failed to find any difference in the viral replication or latency and reactivation between the wt and recombinant virus.

[0193]

The US22 family genes UL36 and UL37 of HCMV have an antiapoptotic function. However, the inventors of the present invention did not observe increased apoptosis in H6R28-infected cells in the present study.

[0194]

Other US22 family genes, such as the MCMV M140 and M141 genes, confer altered cell and tissue tropism. Since the in vivo host tissue range of HHV-6 is broad and since the virus infects various types of cells, it is possible that the HHV-6 US22 family genes contribute to the broad organ tropism of this virus.

[0195]

H6R28 appears to be a useful tool for the study of HHV-6 latency and reactivation. Moreover, in HHV-6 and HHV-7, this large dispensable locus can be a useful site for inserting a large gene, such as a bacterial artificial chromosome (BAC) vector.

[0196]

It is believed that this is the first report of a successful recombinant HHV-6 virus vector, and the invention can provide HHV-6 investigators with a detailed protocol for making it.

[0197]

Note that the similar result to the HHV-6 recombinant virus vector is obtained for HHV-7 recombinant virus vector. To avoid redundancy, no further explanation will be made.

[0198]

The embodiments and concrete examples of implementation discussed in the foregoing detailed explanation serve solely to illustrate the technical details of the present invention, which should not be narrowly interpreted within the limits of such embodiments and concrete examples, but rather may be applied in many variations within the spirit of the present invention, provided such variations do not exceed the scope of the patent claims set forth below.

[BRIEF DESCRIPTION OF THE DRAWINGS]

[0199]

[Figure 1]

A schematic diagram representing a structure of a H6R28 genome.

[Figure 2]

Figure 2 is an electrophoretogram view representing results of PCR amplification that was performed to amplify wild-type (wt) virus DNA and H6R28 virus DNA.

[Figure 3]

Figure 3 a graph representing productive infection of H6R28.

[Figure 4]

A graph showing latency and reactivation of H6R28.

[Figure 5]

Fluorescence micrographs showing EGFP expression in various types of cells.

[Figure 6]

Figure 6 is a schematic diagram and an electrophoretogram showing functions of the HCMV promoter in the latently infected HHV-6.

[Figure 7]

A schematic view for explaining a method for producing recombinant virus vector originating in HHV-7.

[DOCUMENT NAME] ABSTRACT

[ABSTRACT]

[OBJECT] To provide a virus vector by which an exogenous nucleotide sequence can be inserted and easily transferred into a mammalian host cell and a gene encoded by the exogenous nucleotide sequence can be expressed in the host cell, and which has a low risk of pathogenicity and is appropriately usable in gene therapy of mammals.

[MEANS TO ACHIEVE THE OBJECT]

A recombinant vector originating in HHV-6 which has an exogenous nucleotide sequence in a portion corresponding to at least one region selected from the group consisting of U2, U3, U4, U5, U6, U7, and U8 regions of HHV-6; or a recombinant vector originating in HHV-7 which has an exogenous nucleotide sequence in a portion corresponding to at least one region selected from the group consisting of U2, U3, U4, U7, and U8 regions of HHV-7.

[SELECTED DRAWINGS] Figure 1